Genetic Variability of a Forage Bermudagrass Core Collection

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ABSTRACT

Bermudagrass (*Cynodon* sp.) is an important warm-season forage grass for the South and may have value as a bioenergy feedstock. The objective of this study was to measure the genetic relatedness among entries of the *Cynodon* clonal forage bermudagrass core collection and seven commercial forage cultivars using plant phenotype and molecular marker data from amplified fragment length polymorphisms. The collection was assessed for 22 phenotypic traits, including forage quality, plant architecture, growth habit, and ploidy level. Phenotypic variability was preserved in the forage bermudagrass core collection constructed based on 21 phenotypic traits and ploidy levels. The majority of molecular marker polymorphism observed was within phenotypic clusters (89.6%) and within the five ploidy levels (94%), and STRUCTURE analysis indicated significant admixture. Overall, the combined genetic and phenotypic variability found within the bermudagrass core collection will aid in selection of parental crosses for mapping and potential quantitative trait loci discovery and to identify parental lines that may yield greater genetic gain in breeding for important traits.

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Abbreviations: AFLP, amplified fragment length polymorphism; AMOVA, analysis of molecular variance; G #, genotype order number; GSC, genetic similarity coefficient; PCR, polymerase chain reaction; TD, taxonomic dissimilarity; UPGMA, unweighted pair group mean algorithm.

Bermudagrass (*Cynodon* sp.) has been a valuable forage and hay crop in the southern United States since the 19th century (Taliaferro et al., 2004). Highly productive hybrid bermudagrass cultivars have been developed and are now grown as improved pasture on an estimated 10 to 15 million acres in the South. Since the 1930s, breeders have attempted to distinguish genetic traits in bermudagrass important to ruminant nutrition and weight gain, and then select for superior genes in breeding programs. Bermudagrass hybrids with greater yields (Burton, 1943; Burton et al., 1993), better quality for ruminant digestion (Burton, 1972; Burton and Monson, 1984), greater disease tolerance (Burton and Monson, 1988), and higher cold tolerance (Burton and Monson, 1978) have been accomplished through breeding efforts exploiting the great genetic diversity of the bermudagrass collection maintained at Tifton, GA.

For further improvements, breeders require information on genetic diversity for the selection of genetically diverse parents that will maximize genetic gains for desirable traits. Until recently, genetic diversity was measured primarily by phenotypic evaluations of plant material, which required multiple years and locations. Environmental effects reduce differentiation of true genetic

diversity in low-heritability traits. Differential levels of expressed gene products (Dabo et al., 1990; Gonnet, 1993; Grayer et al., 2004; Kaundun et al., 2000; Navarrete et al., 2006; Vermuelen et al., 1991) have also been used to measure diversity. However, gene expression may change under varying locations or harvest times (Brown et al., 2002; Hare, 2002; Lee et al., 2005). Molecular genetic variation is the most recent means of differentiating genotypes and has the advantage of being independent of environmental effects. DNA-amplification fingerprinting (Assefa et al., 1999; Caetano-Anollés et al., 1997), amplified fragment length polymorphism (AFLP) (Zhang et al., 1999), and simple sequence repeats (Karaca et al., 2002) have been used to distinguish between genotypes. To date, there are limited reports on the genetic diversity of international bermudagrass germplasm collections. Fourteen AFLP primer combinations were able to easily distinguish among tetraploid *Cynodon dactylon* (L.) Pers. ($2n = 4x = 36$), diploid *C. transvaalensis* Burtt Davy, and triploid breeding lines (Zhang et al., 1999). Wu et al. (2005) reported significant genetic diversity among 15 *C. transvaalensis* ($2n = 2x = 18$) accessions. Somewhat less diversity was observed among forage bermudagrass genotypes using AFLP (Karaca et al., 2002); however, these authors reached the conclusion that AFLP was the most informative marker type of four surveyed. Wu et al. (2006) reported significant diversity for 132 Chinese *Cynodon* accessions that included primarily turf type material. An extensive assessment of forage type bermudagrass has not been reported in the literature.

A plant introduction nursery of approximately 600 forage bermudagrass accessions is maintained at Tifton, GA. The ploidy levels of these accessions range from $2n = 2x = 18$ to $2n = 6x = 54$. This nursery was assessed for plant growth habit, leaf, stem, and flower heading characteristics, and a core collection was formed by measuring relatedness through CLUSTER analysis of 14 phenotypic traits taken in the summers of 2003 and 2004 along with stand purity and ploidy level (Anderson, 2005). The objective of this follow-up study was to measure and analyze the genetic relatedness among accessions within the bermudagrass core collection using plant phenotypic characteristics and molecular marker data.

MATERIALS AND METHODS
Plant Material, Phenotype, and Ploidy Levels

Plant materials consisted of *Cynodon* clonal accessions maintained at Tifton, GA, as part of a forage core collection, along with seven commercial forage cultivars maintained in breeder plots (Anderson, 2005). Individual accessions were grown in a field nursery at the Coastal Plain Experiment Station, Tifton, GA. Plots of accessions were approximately 2 m² on Tift loamy sand. Phenotypic information was recorded on the individual plants in the nursery for 22 plant traits for cluster analysis. Emergence, dormancy, leaf coarseness, inflorescence emergence and density, homogeneity of stand, plant height, inflorescence

height, lodging, raceme number, raceme length, leaf length, leaf width, internodes, stem thickness, canopy density, head density, regrowth height, and rate of heading were recorded during the summer of 2003 (Anderson, 2005). One plug (5-cm diam.) of each accession was sampled July 2003, grown in 5-cm clay pots in the greenhouse, and transplanted to a second fumigated field on 7 May 2004 (2 m² on Tift loamy sand). Additional data for plant height were recorded after establishment (30 Oct. 2004), as well as after mowing and regrowth in subsequent years (15 June 2005, 3 Sept. 2005, 4 May 2006, 16 Aug. 2007). Dried and ground (1-mm mesh) samples of the 160 core bermudagrass lines harvested in May 2005 and May 2006 were subjected to in vitro dry matter digestibility as described by Tilley and Terry (1963) and averaged over years. Neutral detergent fiber, acid detergent fiber, and acid detergent lignin were determined sequentially (Van Soest et al., 1991) using the Ankom filter bag (Ankom Technology Corp., Fairport, NY) method (Vogel et al., 1999) and sulfuric acid.

Ploidy levels of accessions were determined by obtaining 0.5 cm² of emerging leaf tissue (2–11 May 2003), dicing the tissue with a razor blade, extracting with buffer, staining, and analyzing DNA content with a flow cytometer (Partec Cell Analyzer PAS-III PAR, New Hartford, NY) according to the procedure described by Goldman et al. (2004). Bermudagrass cultivars verified by chromosome counts, T617 (diploid), Tifsport (triploid), 93–166 (tetraploid), and Tifton 10 (hexaploid), were used as standards.

AFLP Analysis

Fresh leaf tissues harvested from three cuttings within each established nursery plot were used for each DNA extraction with the DNeasy plant mini kit from QIAGEN, Inc. (Valencia, CA). The AFLP analyses were performed as described by Vos et al. (1995). Briefly, 100 ng of genomic DNA was double digested with *Eco*RI and *Mse*I restriction enzymes. The AFLP adapters for each enzyme were ligated to the restriction fragments. Ligated DNA was then preamplified with a primer combination to match the adaptor sequences. The *Eco*RI and *Mse*I primer sequences were 5′-GACTGCGTACCAATTC-3′ and 5′-GATGAGTCCTGAG-TAA-3′, respectively. Preamplification conditions were 20 cycles of 94° for 30 sec, 56°C for 1 min, and 72°C for 1 min. Six pairs of selective AFLP primers (Table 1) with *Eco*RI primers labeled with infrared dye were used for selective amplification. Selective amplification conditions used a touchdown protocol, which was 1 cycle of 94°C for 30 sec, 65°C for 30 s, and 72°C for 1 m, followed by 12 cycles of 94°C for 30 sec, 65°C minus 0.7°C cycle⁻¹, and 72°C for 1 m followed by 25 cycles of 94°C for 30 sec, 56°C for 30 s, and 72°C for 1 min. Polymerase chain reactions (PCRs) were conducted in either an MJ Research PTC-200 (Scientific Support, Inc., Hayward, CA) or a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA). A total of 0.5 to 0.8 µL of the selectively amplified PCR products were loaded on a 6.5% (w/v) denaturing gel (LI-COR Inc., Lincoln, NE) and run in 1× TBE buffer at 1500 V, 40 W, and 40 mA at 45°C for 2 h in a LI-COR automated sequencer (LI-COR Inc., Lincoln, NE). IRDye 700–labeled DNA size standard (LI-COR Inc., Lincoln, NE) was loaded on first and last lanes of the gel for molecular weight reference. To ensure accurate scoring and reproducibility, all primer and genotype combinations were replicated three times.

Data Analysis

Polymorphic DNA bands were scored as present (1) or absent (0) for each accession by visual inspection. Data were compiled into a data matrix using Microsoft Excel (Microsoft Corporation, Redmond, WA), and analyzed using NTSYSpc (Numerical Taxonomy System) version 2.2 (Exeter Software, Setauket, NY). Genetic similarity coefficients of pair-wise comparisons among the *Cynodon* core collection were computed based on Jaccard's coefficients (Jaccard, 1908) within the SIMQUAL module. Cluster analysis was performed according to the unweighted pair group mean algorithm (UPGMA) within the SAHN module of the NTSYSpc program. Cophenetic correlation was calculated to measure goodness of fit using the MXCOMP module of NTSYS 2.2. Additionally, bootstrap analysis using 250 replications of the data matrix was performed with the FreeTree program according to the program manual to test for cluster robustness (Hampl et al., 2001). Values >70 were noted.

STRUCTURE software version 2.2 2007 (available from http://pritch.bsd.uchicago.edu/structure.html [verified 19 Apr. 2009]) was utilized to determine the number of structured groups (K clusters) (Falush et al., 2003, 2007; Pritchard et al., 2000). Statistics outlined by Evanno et al. (2005) were utilized in the analysis. Program settings used the admixture ancestry and correlated marker frequency models. The graphs of $L(K)$, as defined by Evanno et al. (2005), along with its variance, and $L'(K)$ were used to determine the number of clusters used for estimating admixtures. The length of burn-in was set at 10,000, followed by 30,000 iterations, and five replications were performed for proposed K values (1–10 tested). Polymorphic information content (PIC), indicating the ability to distinguish among genotypes with each primer combination, was calculated as expected heterozygosity of polymorphic bands according to Powell et al. (1996).

A square-root transformation of phenotypic data with values >10 was utilized to scale all trait scores to fall within a 1-to-10 range of measure. Phenotypic similarity coefficients of pairwise comparisons among the *Cynodon* core collection (Anderson, 2005) were computed as average taxonomic dissimilarity within the SIMINT module. Cluster analysis was performed according to the UPGMA within the SAHN module of the NTSYSpc program. Mantel tests (Mantel, 1967) were completed using the MXCOMP module of NTSYS 2.2 to determine if and to what extent the AFLP marker data were associated with phenotypic data. For the Mantel test, 1000 permutations were calculated. Also, analysis of molecular variance (AMOVA) (Arlequin program) analysis (Excoffier et al., 2006) was performed to determine the percent variation (molecular) within and between phenotypic trait clusters and ploidy levels.

RESULTS
AFLP Results

Reproducibility of the AFLP products used in this study was high. Of the 126 unreadable (too faint or dark) lanes (12.4% of total), the vast majority were a single lane of the three replicates. On six separate occasions, gels were repeated when consistency among the three replicates within the gel varied.

Table 1. Number of total bands, polymorphic bands, percent polymorphic bands, and polymorphic information content (PIC) for each of six amplified fragment length polymorphism selective primer pairs.

Selective amplification primer pairs	Total bands	Polymorphic bands	% Polymorphic bands	PIC
eAAG/mCAT[†]	53	51	96.23	0.45
eACA/mCTT	101	99	98.01	0.29
eACT/mCAT	41	36	87.80	0.25
eAGC/mCAT	78	77	98.7	0.30
eAGG/mCTA	50	40	80.00	0.28
eAGG/mCTT	86	82	95.35	0.15
Total	409	385		
Average	68.17 ± 25.63	64.17 ± 25.48	92.68	0.29 ± 0.10

[†]e = the preamplification primer sequence for *Eco*RI site (5-GACTGCGTACCAATTC) without any selective nucleotides; m = the preamplification primer sequence for *Mse*I site (5-GATGAGTCCTGAGTAA).

Six AFLP selective amplification primer combinations produced a total of 409 bands among the 160 *Cynodon* genotypes, with an average of 68.17 ± 25.63 bands primer combination^{-1} (Table 1). Of the 409 scored bands, 385 were polymorphic, with an average of 64.17 ± 25.48 polymorphic bands primer combination^{-1}. The primer combination eACA/mCTT amplified the largest (101) number of bands, while eACT/mCAT amplified the fewest (41) total numbers and polymorphic bands gel^{-1}. The average PIC value of the six primer combinations was 0.29 ± 0.10, ranging from 0.15 for eAGG/mCTT to 0.45 for eAAG/mCAT.

From the UPGMA analysis, a dendrogram was generated (Fig. 1) with entry number referring to genotype order number (G #) (Table 2). The cophenetic correlation was calculated ($r = 0.86$) as a measure of goodness of fit of the similarity indices. The genetic diversity was relatively high among the plant material in this study. The genetic similarity coefficients (GSCs) given in Fig. 1 are the proportion of matched markers between a given pair of entries among the 160 accessions and cultivars. These values ranged from 0.37 to 1.0. The lowest GSC (0.37) between G# 160 and G# 38, were two *C. dactylon* entries of diploid and tetraploid genome sizes, respectively. The highest GSC (1.0) was detected between G# 19 and G# 20, both of which had a tetraploid genome size. These two accessions had been maintained side by side for an extended period of time in the nursery and cross contamination was suspected, which these results supported. Bootstrap values of the GSC cluster analysis tended to be low, with few cluster values >70 (43 of 150 total clusters), which are shown in Fig. 1. All clusters not showing a value are <70.

The estimated log probability of data [$L(K)$] resulting from Bayesian cluster analysis generally improved from $K = 1$ to $K = 8$, with substantial rates of gain up to $K = 5$ (Table 3). At $K = 5$ the $L(K)$ began to plateau, and inferred ancestry coefficients (Fig. 2) generally correlated well with groups supported by UPGMA
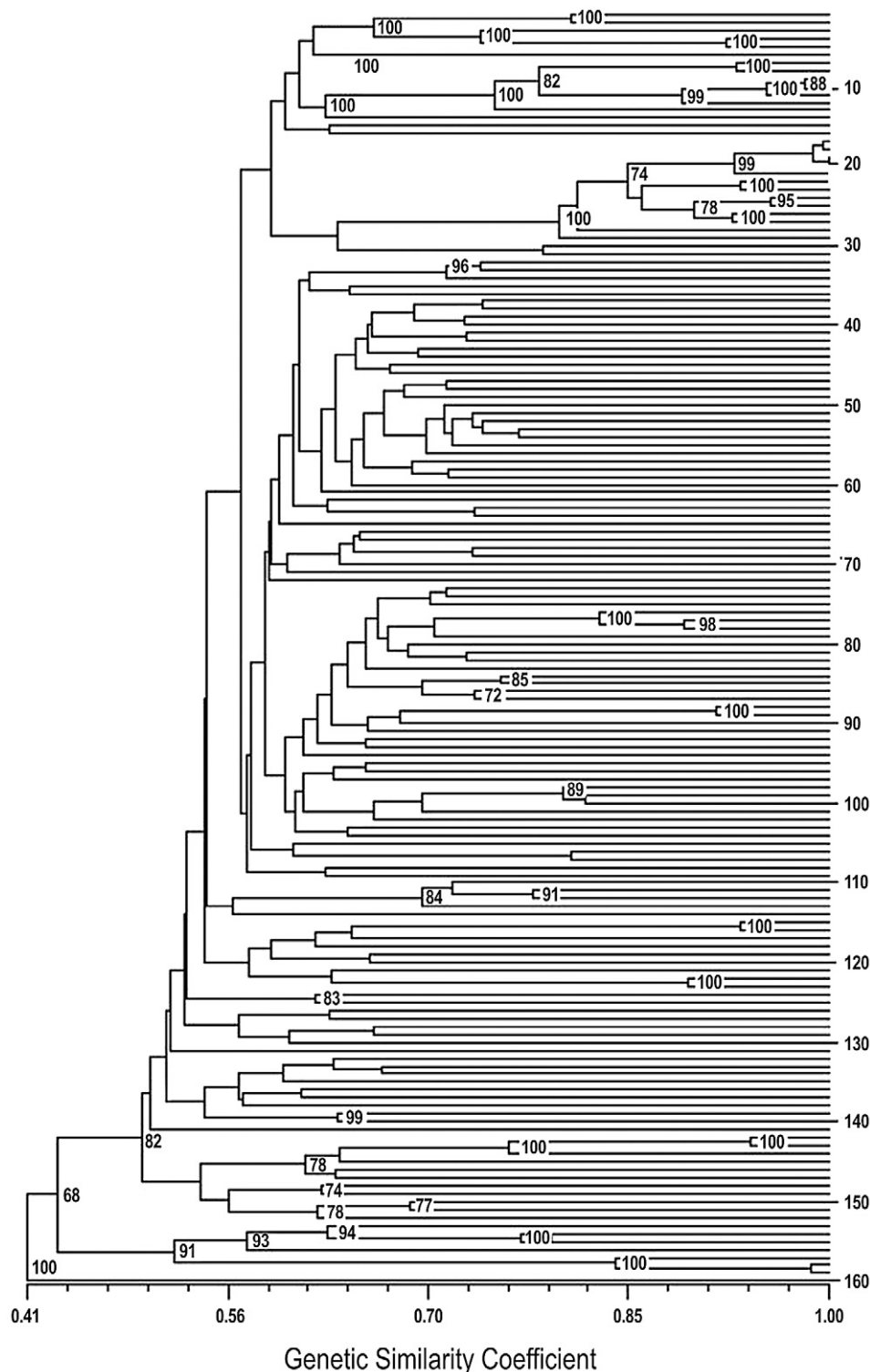
Figure 1. Dendrogram of 160 *Cynodon* clonal accessions (including seven commercial forage cultivars) maintained at Tifton, GA, as part of a forage core collection, produced by unweighted pair group mean algorithm clustering methods based on the genetic similarity matrix (genetic similarity coefficient) derived from 409 markers. Bootstrap values are displayed. Numbers correspond to genotype order (Table 2).

bootstrap confidence levels (Fig. 1). The overall proportion of membership in each of the five clusters was 48.0% (A—yellow), 6.1% (B—blue), 10.3% (C—pink), 17.1% (D—red), and 18.4% (E—green) in Clusters 1 through 5, respectively, but admixture was observed among all five types (Fig. 2 and Table 2). Results from the STRUCTURE analysis generally correlated well with the small clusters of entries with high bootstrap values from the dendrogram (Fig. 1). However, the groupings over large numbers of entries were not consistent, as evidenced by clusters and admixtures with A, C, D, and E of the STRUCTURE analysis spread across a large portion of the dendrogram (Fig. 1 and 2).

Phenotypic Results

The taxonomic dissimilarity (TD) among the 152 accessions ranged from 0.49 to 0.96 (Fig. 3). The lowest TD (0.49) was observed between G# 19 and G# 20. Phenotypic similarity between these accessions corresponded to genotypic results obtained from AFLPs (Fig. 1). The highest TD (4.96) was detected between G# 19, a *C. dactylon,* and G# 150, a *Cynodon* spp. entry yet to be taxonomically defined. Ploidy levels for these two entries were tetraploid and diploid, respectively.

Cluster analysis based on taxonomic dissimilarity separated the *Cynodon* accessions into two distinct groups having dissimilarity values of 2.5 or less within each cluster: A and B (Fig. 3 and Table 2). Cluster A contained 106 entries consisting of mostly fine-stemmed short genotypes, and Cluster B contained 46 entries consisting of coarse-stemmed tall genotypes (Table 4). The phenotypic surveys of the variation patterns and groupings of this material did not tend to cluster according to ploidy or major geographic regions. The Mantel test resulted in a significant ($P \leq 0.001$) correlation of the phenotypic and genotypic matrices with an $r = 0.42$. Though significant, the low

Table 2. Genotype order number from Fig. 1 and 2, core collection number, genotype cluster from Fig. 2, plant identification (PI no.), species, origin, ploidy level, phenotype order from Fig. 3, phenotype cluster, and phenotypic scores for forage characteristics (leaf–stem coarseness, plant height) of 168 *Cynodon* accessions.

Genotype order no.	Core collection no.	Genotype cluster–admixture[†]	PI no.	Species–cultivar	Origin[‡]	Ploidy level	Phenotype order	Phenotype cluster	Leaf–stem coarseness	Plant height
										cm
1	4	A		Coastal[§]		4			3	46
2	43	A/C/E	224692	*C. dactylon*	South Africa	4	65	A	4	43
3	98	A/B/E	291576	*C. dactylon*	Salisbury, Rhodesia	4	83	A	3	50
4	108	A/B/E	287246	*C. dactylon*	Hyderabad, India	5			4	58
5	109	A/B/E		Callie		4			4	60
6	82	A		*Cynodon* spp.	Rienzi, MS	5	106	A	3	18
7	99	A/B	290901	*C. dactylon*	Pretoria, S.A.	4	123	B	4	52
8	100	A/B	294467	*C. dactylon*	South Africa	4	124	B	4	57
9	104	B	290872	*C. nlemfuensis*	Johannesburg, S.A.	4	126	B	4	53
10	105	B	290812	*C. nlemfuensis*	Middleburg, S.A.	4	128	B	4	58
11	106	B	290664	*C. hirsutus*	Pretoria, S.A.	4	149	B	4	54
12	107	B	295114	*C. dactylon*	Rhodesia	4	150	B	4	52
13	112	A/B	290813	*C. nlemfuensis*	Edenburg, S.A.	4	151	B	4	55
14	129	A/B/E	308193	*C. dactylon*	Nigeria (Chedda via Burton)	4	141	B	3	50
15	102	A	290891	*C. hirsutus*	Pretoria, S.A.	4	26	A	3	41
16	139	A/C/E	22610	*C. plectostachyus*	Rhodesia	4	152	B	4	57
17	2	C		Coastcross I[§]		4			5	52
18	3	C		Coastcross II[§]		4			5	53
19	10	C	204438	*C. dactylon*	Turkey	4	107	B	5	46
20	11	C		Coastcross I		4	108	B	5	52
21	19	C		Coastcross II		4	110	B	5	49
22	158	C	316536	*C. dactylon*	Vienna, Austria	4	135	B	4	57
23	159	C	316416	*C. dactylon*	Dorstadt, Switzerland	4	139	B	4	56
24	166	C	316418	*C. dactylon*	Saxon, Switzerland	4	133	B	4	52
25	167	C	316536	*C. dactylon*	Vienna, Austria	4	136	B	4	51
26	169	C		African Star (23-20)		4	138	B	4	53
27	170	C		African Star (24-17)		4	137	B	4	56
28	152	C		Winterhardy Bermuda	From Purdue Univ.	4	131	B	5	53
29	138	A/C	255457	*C. plectostachyus*	Kenya	4	132	B	4	40
30	76	A/D/E	287154	*C. dactylon*	Iyerpadi, India	3	11	A	1	30
31	78	A		*C. dactylon*	F$_1$ hybrid	4	134	B	4	47
32	5	A/E		T 292[§]		4			2	40
33	70	A	290884	*C. dactylon*	Pretoria, S.A.	4	67	A	3	38
34	86	A/D	290884	*C. dactylon*	Pretoria, S.A.	4			2	41
35	69	A	290666	*Cynodon* spp.	Pretoria, S.A.	4	88	A	3	35
36	111	A	291965	*C. dactylon*	Kitale, Kenya	4	29	A	2	34
37	22	A	290879	*C. dactylon*	Pretoria, S.A.	4	80	A	1	42
38	101	A	290877	*C. dactylon*	Pretoria, S.A.	4	71	A	2	37
39	30	A	224568	*C. dactylon*	Rhodesia	4	81	A	3	39
40	42	A	225809	*Cynodon* spp.	Africa	4			2	42
41	27	A/C	291587	*C. dactylon*	Salisbury, Rhodesia	4	63	A	3	36
42	28	A	290667	*C. dactylon*	Pretoria, S.A.	4	59	A	2	32
43	24	A	289926	*C. magennisii*[¶]	Johannesburg, S.A.	4	48	A	2	33
44	26	A	291614	*C. dactylon*	Kroonstad, S.A.	4	64	A	3	45
45	39	A	292246	*C. arcuatus*[¶]	Los Baños, Philippines	4	66	A	4	42
46	40	A/D	289930	*C. dactylon*	Durban, S.A.	4	72	A	3	41
47	44	A		*Cynodon* spp.	Sandhill Test #6	4	28	A	2	40
48	77	A/C/E		Breeding line	Sandhill Test #9	4	60	A	3	43
49	132	A	289613	*C. arcuatus*	Noisse Be, Malagasy	4	86	A	3	42
50	135	A	291744	*C. dactylon*	Pretoria, S.A.	4	74	A	2	41

Table 2. Continued.

Genotype order no.	Core collection no.	Genotype cluster–admixture[†]	PI no.	Species–cultivar	Origin[‡]	Ploidy level	Phenotype order	Phenotype cluster	Leaf–stem coarseness	Plant height
										cm
51	142	A	291161	*C. dactylon*	Pretoria, S.A.	4	44	A	2	32
52	143	A	291158	*C. dactylon*	Pretoria, S.A.	4	75	A	2	38
53	144	A	291155	*C. dactylon*	Bloemhoff, S.A.	4	104	A	3	44
54	146	A	291150	*C. dactylon*	Sannieshof, S.A.	4	68	A	2	41
55	147	A	291749	*C. dactylon*	Pretoria, S.A.	4	89	A	1	32
56	141	A	291164	*C. dactylon*	Mooi River, S.A.	4	43	A	2	30
57	148	A	291712	*C. dactylon*	Pretoria, S.A.	4	70	A	2	39
58	149	A	291176	*C. dactylon*	Pretoria, S.A.	4	87	A	3	36
59	168	A	316536	*C. dactylon*	Vienna, Austria	4	105	A	1	49
60	60	A	291575	*C. bradleyi*[¶]	Salisbury, Rhodesia	4	77	A	2	37
61	95	E	288217	*C. coursii*	Lake Alaotra, Malagasy	4	69	A	2	45
62	15	A	291588	*C. dactylon*	Salisbury, Rhodesia	4	57	A	3	34
63	46	A	291584	*C. dactylon*	Salisbury, Rhodesia	3	78	A	3	35
64	74	A/D	291958	*C. dactylon*	Kitale, Kenya	4	79	A	2	36
65	20	A	290899	*C. dactylon*	Pretoria, S.A.	4	27	A	3	32
66	58	A/E	287139	*C. barberi*[¶]	Coimbatore, India	3	96	A	4	46
67	64	A/E	288222	*C. coursii*	Maharidaza, Malagasy	3	100	A	4	42
68	81	A/D/E	287155	*C. dactylon*	Kavarkal, India	4	97	A	3	55
69	91	A	287156	*C. dactylon*	Kavarkal, India	4	98	A	3	49
70	63	A	None		Johannesburg, S.A.	4	85	A	3	40
71	126	A/E	289716	*C. coursii*	Lake Alaotra, Malagasy	4	45	A	3	42
72	23	A	290901	*C. dactylon*	Pretoria, S.A.	2	46	A	2	34
73	1	A/C/E		Alicia[‡]		4			2	35
74	12	A/D		*C. dactylon*	NK-37 (Arizona)	4	25	A	4	39
75	21	A/D	292602	*C. dactylon*	Kenya	4	35	A	2	27
76	13	A/D	286583	*C. dactylon*	Bombay, India	4	31	A	3	36
77	25	A/D	292142	*C. dactylon*	Dabala, Ghana	4	53	A	2	29
78	29	A/D	286582	*C. barberi*	Bombay, India	4	32	A	2	30
79	41	A/D	292544	*C. dactylon*	Pakchong, Thailand	4	82	A	2	36
80	125	A/D	266768	*C. dactylon*	Iran	4	93	A	2	33
81	127	A/D	301862	*Cynodon* spp.	Australia	4	92	A	3	38
82	128	A/D	301858	*Cynodon* spp.	Australia	4	73	A	2	43
83	73	A/D	291193	*C. polevansii*[¶]	Delareyville, S.A.	4	37	A	1	29
84	48	A/D	289748	*C. polevansii*	Barberspan, S.A.	3	21	A	2	29
85	68	A/D	289750	*C. polevansii*	Barberspan, S.A.	4	52	A	1	24
86	83	A/C/D	290885	*C. dactylon*	Pretoria, S.A.	4	61	A	3	32
87	84	A/D	293641	*C. dactylon*	Kitale, Kenya	4	90	A	2	30
88	87	A		Breeding line	Tifton 79-16	4	41	A	2	28
89	88	A/D	289917	*C. nlemfuensis*	Johannesburg, S.A.	5	125	B	4	54
90	97	A/D	290661	*C. dactylon*	Pretoria, S.A.	4	33	A	2	33
91	160	A/D	316419	*C. dactylon*	Sierre, Switzerland	4	23	A	2	28
92	50	A/D	289930	*C. dactylon*	South Africa	3	40	A	1	23
93	55	A/D	292228	*C. dactylon*	Koforidua, Ghana	4	39	A	2	33
94	153	A/D		Winterhardy Bermuda	From Purdue Univ.	4	36	A	1	28
95	96	A/C	224693	*C. dactylon*	Africa	4	58	A	2	42
96	119	A/C	288221	*C. coursii*	Ampasikely, Malagasy	4	99	A	3	46
97	120	A/C/D	212293	*C. dactylon*	Afghanistan	4	102	A	3	33
98	113	A/D		Breeding line		4	12	A	2	25
99	117	A/D	203456	*C. dactylon*	Turkey	5	101	A	2	36
100	121	A/D		Callie		4	62	A	3	32

Table 2. Continued.

Genotype order no.	Core collection no.	Genotype cluster–admixture[†]	PI no.	Species–cultivar	Origin[‡]	Ploidy level	Phenotype order	Phenotype cluster	Leaf–stem coarseness	Plant height
										cm
101	122	A/D	293606	*C. nlemfuensis*	Nakuru, Kenya	4	34	A	2	36
102	123	A/B		Tifton 44		4	42	A	3	37
103	115	A	223357	*C. dactylon*	Iran	4	84	A	2	32
104	116	A/D	206553	*C. dactylon*	Greece	4	30	A	2	34
105	31	A/E		*Cynodon* spp.	Arizona Seed lot 12058	3	111	B	5	35
106	157	A/E	315904	*C. dactylon*	Hundszahangras, Berlin-Tegel	4	94	A	2	36
107	161	A/E	316420	*C. dactylon*	Lugano, Switzerland	4	95	A	1	40
108	9	A	224148	*Cynodon* spp.	South Africa	4	1	A	2	28
109	103	A	290895	*C. dactylon*	Pretoria, S.A.	3	4	A	1	24
110	33	A/D	290902	*C. dactylon*	Pretoria, S.A.	4	38	A	2	21
111	51	A/D	290895	*C. dactylon*	Pretoria, S.A.	3	54	A	2	29
112	85	A/D	290887	*C. dactylon*	Pretoria, S.A.	4	55	A	2	20
113	71	A	290885	*C. dactylon*	Pretoria, S.A.	3	56	A	2	24
114	62	A/D/E	224129	*C. dactylon*	Pretoria, S.A.	3	19	A	2	24
115	34	C/D/E	225809	*Cynodon* spp.	Africa	4	115	B	5	59
116	35	C/D/E	292059	*C. dactylon*	Dar es Salaam, Tanzania	2	130	B	5	59
117	163	C/D/E		Coffee 210		4	121	B	3	43
118	75	A		Breeding line	77-59	5	117	B	4	50
119	49	A/E	225591	*C. dactylon*	Tanzania	4	122	B	5	56
120	56	A/D/E		Breeding line	77-57	4	140	B	3	48
121	57	E		Breeding line	77-58	4	116	B	5	66
122	155	E	316510	*C. dactylon*	Germany	4	119	B	5	69
123	156	E	316507	*C. dactylon*	Germany	4	120	B	4	59
124	54	A	289749	*C. polevansii*	Barberspan, S.A.	5	2	A	1	29
125	90	A/D	290660	*C. dactylon*	Pretoria, S.A.	5	50	A	2	27
126	16	A/E		Coastcross III		4	114	B	4	39
127	165	A/D/E		*C. dactylon*	Miami, FL	4	91	A	3	33
128	80	A/D		*Cynodon* spp.	St. Louis, MO	5	10	A	2	15
129	164	D/E		*C. dactylon*	Miami, FL	4	18	A	2	16
130	154	A/D/E	320876	*C. dactylon*	Italy	5	8	A	2	22
131	36	A	290896	*C. dactylon*	Pretoria, S.A.	4	3	A	1	14
132	7	E		Tifton 85[§]		5			5	63
133	6	A/D		*C. dactylon*	Pretoria, S.A.	6			5	65
134	124	E		Tifton 68		6	113	B	5	59
135	140	E		Breeding line	78-9	6	148	B	5	41
136	52	E		Breeding line	77-29	5	146	B	4	56
137	114	E		Breeding line	80-12	4	147	B	3	36
138	118	E		Breeding line	80-23	4	145	B	4	48
139	59	E	255450	*C. nlemfuensis*	Kenya	3	127	B	5	54
140	61	E		Tifton 84		4	24	A	5	32
141	53	A/E		*Cynodon* spp.	Michigan State Univ. (Milo Tesar)	4	9	A	2	22
142	17	D	292247	*C. dactylon*	Los Baños, Philippines	2	15	A	2	17
143	18	D	292601	*C. dactylon*	Kenya	4	17	A	3	16
144	47	D	292252	*C. dactylon*	Bataan, Philippines	3	16	A	2	19
145	45	D	288043	*C. dactylon*	Aliyar Dam, India	3	7	A	2	23
146	65	D	287708	*C. dactylon*	Narsampet, India	3	51	A	1	17
147	92	A		*Cynodon* spp.	Sandhill Test #7	4			2	26
148	66	D/E	291957	*C. dactylon*	Kitale, Kenya	3	22	A	3	27
149	67	D/E	292509	*C. dactylon*	Honshu, Japan	5	6	A	2	17

Table 2. Continued.

Genotype order no.	Core collection no.	Genotype cluster–admixture[†]	PI no.	Species–cultivar	Origin[‡]	Ploidy level	Phenotype order	Phenotype cluster	Leaf–stem coarseness	Plant height
										cm
150	72	A/D	292253	*Cynodon* spp.	Baguio City, Philippines	2	13	A	1	9
151	79	A/E	224128	*C. dactylon*	Kitale, Kenya	4	103	A	2	42
152	89	A/D	291962	*C. dactylon*	Kitale, Kenya	3	14	A	1	14
153	14	E	293616	*C. dactylon*	Kitale, Kenya	2	112	B	5	63
154	32	E	292143	*C. plectostachyus*	Ohawu, Ghana	4	143	B	5	50
155	37	E	289929	*Cynodon* spp.	Durban, S.A.	2	144	B	5	48
156	94	D	255456	*Cynodon* spp.	Cross	2			4	62
157	130	E		*C. dactylon*	Okla. Acc. no. 9214	4	129	B	4	52
158	136	E	293653	*C. plectostachyus*	Kitale, Kenya	4	118	B	4	45
159	137	E	292057	*C. plectostachyus*	Mt. Makulu, N. Rhodesia	4	142	B	4	36
160	38	E	288044	*C. dactylon*	Pollachi, India	2	20	A	3	28
	MSD[#]									8

[†]From Fig. 3: A (yellow), B (blue), C (pink), D (green), and E (red)—predominant cluster or admixture results from STRUCTURE analysis.

[‡]S.A. = South Africa, Okla. Acc. = Oklahoma Accession.

[§]Check entries (commercial entries) from breeder's nursery.

[¶]Authorities: *Cynodon arcuatus* J. Presl; *C. barberi* Rang. & Tad.; *C. bradleyi* Stent; *C. ×magennisii* Hurcombe; *C. polevansii* Stent.

[#]MSD = minimum significant difference.

correlation coefficient indicates extensive genetic variability within phenotypically similar clusters. This was supported by AMOVA analysis, which indicated that only 10.36% of the genetic polymorphism was apportioned between the two phenotypic clusters and only 6.0% among the ploidy levels.

DISCUSSION

It is clear that the phenotypic dendrogram separated coarse-stemmed tall entries from fine-stemmed short entries (Fig. 3 and Table 4). Variability among entries existed for all other traits within these clusters as was reported by Anderson (2005). Most of the observed genetic polymorphism

Table 3. Mean estimated log probability of data [*L*(*K*)] under exhaustive sampling (mean over five runs), mean variance, standard deviation of *L*(*K*) of STRUCTURE program, and mean difference for successive *L*(*K*) in model.

Value of *K*	Mean *L*(*K*)[†]	Mean variance	SD *L*(*K*)	Mean *L*′(*K*)[‡]
1	−42,438	2,555	51	–
2	−40,311	3,603	146	2127
3	−38,109	4,047	36	2203
4	−36,754	4,077	190	1355
5	−35,657	4,225	63	1097
6	−35,218	4,485	38	449
7	−35,335	5,884	1266	−118
8	−34,560	5,190	74	775
9	−36,201	9,226	1953	−1641
10	−40,448	18,298	5699	−4247

[†]*L*(*K*) = log likelihood of the data from STRUCTURE software program.

[‡]*L*′(*K*) = *L*(*K*) − *L*(*K* − 1).

was within phenotypic groups and ploidy levels as evidenced by the AMOVA analysis and Mantel test.

The genetic variability among all accessions is substantial based on the high degree of polymorphism among AFLPs (Table 1) and as shown by the dendrogram and admixture observed from the Pritchard STRUCTURE analysis (Fig. 1 and 2). Previous studies by Karaca et al. (2002) of 31 forage bermudagrass cultivars, Wu et al. (2005) of 14 *C. transvaalensis* accessions, and Wu et al. (2006) of 119 accessions, indicated significant polymorphism using AFLPs. These studies gave us reason to expect significant variability within the bermudagrass forage core collection. Based on the GSA scores of 0.56 to 1.0 in this study, the genetic diversity available within the bermudagrass core collection is extensive.

The groupings and admixture from the STRUCTURE analysis appear to be a more constructive depiction of AFLP marker distribution than the dendrogram generated by NTSYSpc. As Evanno et al. (2005) pointed out, the STRUCTURE analysis allows for quantification of how likely each individual is to belong to a group. The analysis allows for assignments of entries into clusters or admixtures and is appropriate for population sizes of 10 or more using at least 100 AFLP markers (Evanno et al., 2005). The program was modified to account for dominant markers such as from AFLPs (Falush et al., 2007).

In our study, the STRUCTURE output suggested 5 as the most informative *K* level. With *K* set at 5, four of the five clusters fall within areas of high bootstrap values from the NTSYSpc analysis, which are genotype order numbers 7 to 13 (bootstrap 100), 17 to 29 (bootstrap 100),
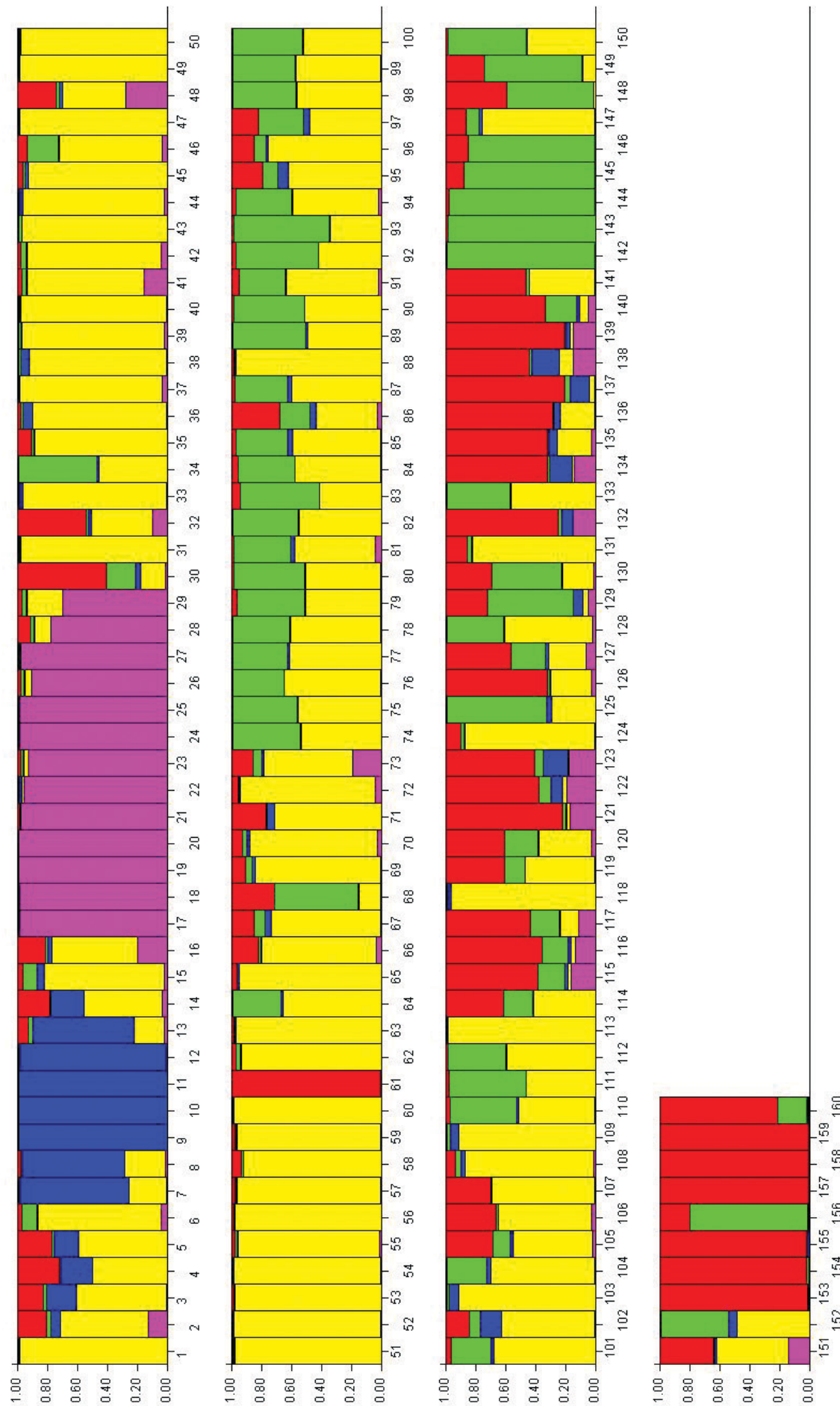
Figure 2. Graphic representation of the individual ancestry coefficients (percentage of alleles) of 160 *Cynodon* accessions maintained at Tifton, GA, that are attributed to one of five clusters (Clusters 1–5 represented by yellow [A], blue [B], pink [C], green [D], and red [E], respectively) as determined by STRUCTURE software version 2.2 2007. Numbers correspond to genotype order (Table 2).

142 to 146 (bootstrap 78), and 153 to 159 (bootstrap 91) with the exception of entry 156, which exhibited admixture (Fig. 1 and 2). Though $K = 5$ appeared appropriate for this study, the observed extensive admixture confirms the difficulty in definitively clustering larger groups of accessions. Many accessions within the collection were representative of admixture with Cluster A (Table 2; represented by yellow in the STRUCTURE analysis) (Fig. 2) contributing to nearly half of the overall structure. The percentage of cluster likelihoods depicted in Fig. 2 was used to assign bermudagrass entries into clusters or admixtures presented in Table 2. The admixtures better explain the lack of high bootstrap values with the NTSYSpc analysis. It is also possible to observe similarities within and among groups in Fig. 2 that cannot be observed from the dendrograms (Fig. 1). Furthermore, the STRUCTURE analysis showed discrepancies from the dendrogram (i.e., G# 30, 61, 68, 118, 156) (Fig. 1 and 2), exposing faults to using genetic trees to identify clusters. Genetically distinct entries can thus be selected from the STRUCTURE output (Fig. 2) for further evaluation, for selection of parents to generate mapping populations to aid with identification of molecular markers useful for marker-assisted selection, or for restructuring of the original core collection.

In previous studies, clustering corresponded with geographic regions and phenotype (Wu. et al., 2005, 2006). However, in the accessions of this study the variation patterns and groupings did not tend to cluster according to ploidy or phenotypic variation. This is most likely due to the contrived nature of the core collection to capture a wide range of phenotypic variability (Anderson, 2005) while maintaining the proper representation of ploidy levels. Entries from this study were an accumulation of germplasm collected from different parts of the world at different times over the past century, whereas each of the previous studies had tighter population structure either by specific species or geographic location.

There were a number of duplicates or near duplicates included within the core collection analysis. Two sources of 'Coastcross I' (G# 17 and 20) and 'Coastcross II' (derived from mutation of Coastcross I) (G# 18 and 21) clustered very closely together, both genetically (Fig. 1: G# 17–21; Fig. 2: Cluster C) and phenotypically (Fig. 3: G# 107–110). It is evident that a nearby plot within the nursery (G# 19) had been invaded by Coastcross II. The genotypic coefficient of similarity was very high and the phenotypic coefficient of distance was small for these genotypes, indicating duplicity. Also, 'Tifton 68' (G# 134) and 'Tifton 85' (G# 132) clustered very closely with each other (Fig. 1 and Table 2). Tifton 85 (Burton et al., 1993) is a hybrid of Tifton 68 (Burton and Monson, 1984) and PI 290884 (G# 32 and 33). Tifton 85 was released based on yield and ruminant digestibility characteristics similar to Tifton 68 but with much greater
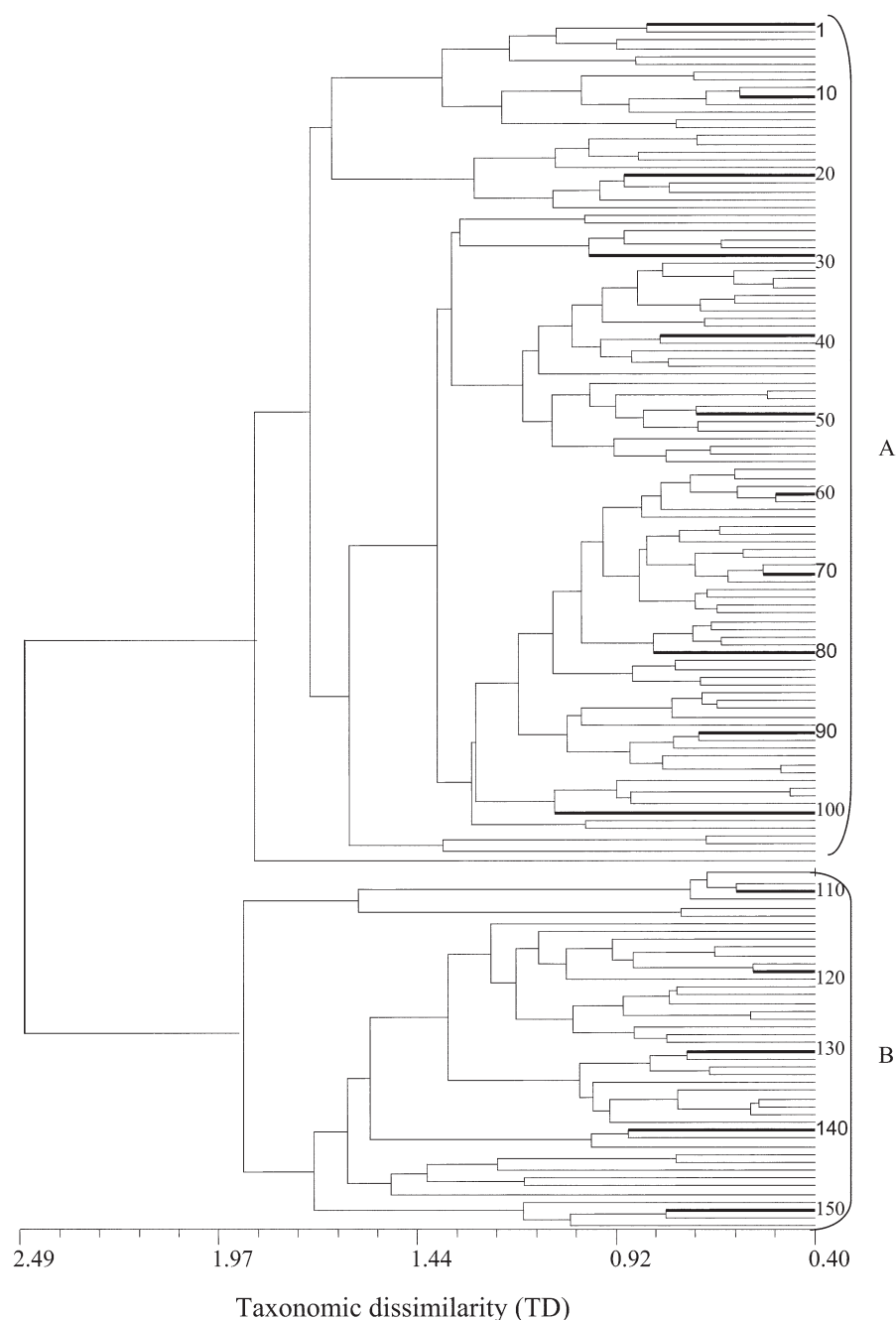

Figure 3. Dendrogram of 152 *Cynodon* accessions maintained at Tifton, GA, produced by taxonomic dissimilarity analysis of 22 phenotypic plant traits used in the development of a bermudagrass core collection. Numbers correspond to phenotype order (Table 2). A and B are designated phenotypic clusters.

cold tolerance (from PI 290884). The relatively high genetic distance between Tifton 85 and PI 290884 supports the observed wide phenotypic difference except for cold tolerance.

The original passport data for entries in this study specified numerous species (Table 2). The species were not easily differentiated within the STRUCTURE analysis (Fig. 2) or phenotypic clustering (Fig. 3). This is most likely due to misnaming of these accessions before the revision of the genus in the 1960s by Harlan et al. (1970). The plant introductions were collected and established at Tifton, beginning in the 1940s and, thus, since then contamination among nursery plots may have also occurred. There was a great deal of confusion within the literature as to taxonomic classifications before 1970 (Harlan, 1970). For example, *C. plectostachyus* (K. Schum.) Pilg. is described by Harlan to be diploid, but the accessions in this core collection were tetraploid. Also, *C. hirsutus* (Stent) de Wet & J. R. Harlan and *C. coursii* (A. Camus) J. R. Harlan & de Wet are considered botanical varieties rather than species by Harlan (Harlan et al., 1970). Some of the coarse "stargrass" accessions previously classified as *C. dactylon* were reclassified by Harlan as *C. nlemfuensis* Vanderyst (Taliaferro et al., 2004). PI 255450 (G# 139) and PI 293606 (G# 101) are *C. nlemfuensis* parents of Tifton 68. Other accessions genetically similar to PI 255450 and Tifton 68 (Fig. 2), listed as *C. dactylon* or *C. plectostachyus* within the B phenotypic cluster (Fig. 3), fit classification characteristics of *C. nlemfuensis* (Harlan et al., 1970). Reclassification of the core collection should be made easier by using the phenotypic clustering and the STRUCTURE analysis performed herein.

Genetic variation among accessions was discovered by the AFLP analysis that was not observed from phenotypic evaluations. Genotype 160 was genetically distinct in the dendrogram (Fig. 1) from all other entries; however, it clustered phenotypically within the large A cluster (Fig. 3) that contained short-stature and fine-stemmed accessions. The phenotypic cluster of fine-stemmed shorter accessions (Fig. 2: phenotype cluster A) generally corresponded with genotype clusters and admixtures with A and D (Fig. 2 and Table 2), and the coarse, tall accessions (phenotype cluster B) with genotypic clusters B, C, and E. However, there were numerous exceptions and a great deal of genetic admixture among many accessions. Coarse entries from genetic clusters B, C, or E should be considered potential parents with coarse entries from clusters or admixtures with A and D (for example G# 75, 88, or 151) for incorporation of additional genes to confer potential hybrid vigor for tall growth habit.

Though AMOVA resulted in very low genotypic variation between phenotypic clusters (10.36%), the Mantel test (Mantel, 1967) indicated significant ($P \leq 0.001$)

Table 4. Distribution of *Cynodon* accessions within Clusters A and B of the phenotypic tree for stem and leaf coarseness and plant height (average of six measurements).

Cluster	Stem and leaf coarseness				
	Very fine	Fine	Moderately coarse	Coarse	Very coarse
A	15	53	31	5	2
B	0	0	4	25	18
	Average plant height				
	<21 cm	21–30 cm	31–40 cm	41–50 cm	51–60 cm	>60 cm
A	12	27	40	22	1	
B	0	0	5	12	25	3

correlation between phenotypic and genotypic matrices (r = 0.42). While this represents a low correlation between genotypic and phenotypic information, it is higher than the r = 0.12 Johnson et al. (2007) found in safflower (*Carthamus tinctorius* L.). Reed and Frankham (2001) examined 70 data sets and found a mean correlation coefficient of 0.36 between molecular and phenotypic variation. Reed and Frankham (2001) attributed the low correlations to variation in molecular markers and the result of genetic drift contributing to variation, whereas adaptive phenotypic variation would be driven more by selection. This would suggest that more of the polymorphic loci identified in this study may be contributing to differentiation of phenotypic factors than shown in previous studies, which bodes well for potential use of these accessions for association mapping.

In conclusion, the combined genetic and phenotypic analyses of the accessions within the bermudagrass core collection can now be used to design parental crosses that can maximize genetic polymorphism for traits such as growth habit and quality. Additional analysis of the collection for important forage or biofuel traits such as stress tolerance, cell wall components, and digestibility will further differentiate accessions, and when combined with molecular marker data, can be used directly for association mapping or to assist in the selection of parents for structured population development.

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